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CERTIFICATE

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I hereby certify that annexed is a true copy of the Provisional Specification as filed on 24 April 2003 with an application for Letters Patent number 525521 made by UNIVERSITY OF WAIKATO and UNIVERSIDAD DE CHILE.

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Dated 29 April 2004.

Neville Harris

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Commissioner of Patents, Trade Marks and Designs

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NEW ZEALAND PATENTS ACT 1953

PROVISIONAL SPECIFICATION

ISOLATION AND USE OF WHITE ROT FUNGI

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We, UNIVERSITY OF WAIKATO, constituted under the University of Waikato Act 1963 of Hillcrest Road, Hamilton, New Zealand, and UNIVERSIDAD DE CHILE of Alameda 1058, Santiago, Chile, do hereby declare this invention to be described in the following statement:

Technical Field

The present invention relates to the development of certain fungi useful in the enhancement of wood or wood products quality. More particularly, but not exclusively, it relates to a process for the development of a composition comprising or including white rot decay fungi (WDF) from an inoculum to solid wood, particularly but not exclusively logs, chips, chips and/or sawdust of most specifically but not exclusively ***Pinus radiata,*** and ***Eucalyptus spp***, such WDF being useful to improve cellulosic pulp production.

Background

The pulp industry is based mainly on chemical processes of digestion. One example is the kraft system which uses wood as a raw material, from natural forests or from plantations, such as ***Pinus radiata,*** and ***Eucalyptus spp***. This digestion process aims for a decrease in, or elimination of lignin in order to obtain cellulosic pulps.

Such chemical digestion processes are frequently discussed due to the negative environmental impact caused by the resulting effluents and the contamination derived from them. For this reason alternative processes of chemical digestion, which may have diminished environmental impact are begin sought, such as processes which require less reagent.

One route to a more environmentally favourable process is the use of biotechnology as part of the chemical digestion processes - for instance the application of white rot decay fungi (WDF).

Fungi are biological organisms which can be described as heterotrophs and eucaryotic which have enzymatic systems that allow them to depolymerize organic substances, previously synthesized by chlorophylic plants. From that point of view and in the environment of the forest products pathology, the lignin decay fungi in the wide sense cause on wood diverse alterations like molds, stains, and decay (hereinafter referred to essentially as decay).

Three decay types are recognized on wood:

- brown rot decay (holocellulose depolymerization) by means of enzymes that act via hydrolytic enzymatic action,
- white rot decay (depolymerization of all the structural components of the woody cellular wall: mainly lignin and holocellulose, in secondary form), by means of hydrolytic and oxidative enzymes, and
- soft rot decay that is defined by its action of hydrolytic and oxidative enzymes on the substrate S2 of the secondary wall.

Lignin is a polymer of high complexity. It is a polyphenolic substance formed by three types of phenyl-propane units that form an aromatic heterogeneous compound and constitute 20 to 30% of the cellular wall. The biggest proportion of lignin is located in the middle lamella. Together with the pectins, lignin is also in the primary cell wall together with holocellulose, completing a list of structural and resistance elements. Topographically, it is located mainly in the cellular vertexes, in form of units denominated G lignin (units of guacylics alcohols) that are highly condensed and of difficult chemical digestion.

While the use of white rot decay fungi (WDF) for delignification of wood has been reported[1,2,3,4,5,6,7,8,9], there has been no practical commercial application of the technology, mainly due to the accompanying characteristic of cellulose degradation by the fungi.

Object of the Invention

It is amongst self evident object of the invention to provide a means of using white rot decay fungi to treat wood which will overcome the abovementioned disadvantages, and/or to provide an application of a composition comprising or including white rot decay fungi as an inoculum for wood which is to be treated by chemical process to obtain pulps, and/or which will at least provide the public with a useful alternative.

Summary of the Invention

In a first aspect of the present invention there is provided **a method of isolating white rot decay fungi** which will have a positive effect on wood and/or wood products and/or

ood processing by providing one or both of a lignocelluslosic and/or extractives decrease in the wood, the method comprising or including the steps of:

1) collection of white rot decay fungi (whether from nature or otherwise);
2) optionally culturing the collected fungi;
3) preparation of a solid cultivation of the isolated fungi;
4) subjecting the cultivated fungi to a selection process to distinguish desired fungi from unwanted fungi, wherein the selection process includes or comprises subjecting the cultivated fungi to both:
   a) a test to establish production of oxidative enzymes; and
   b) a test to establish the resistance of the cultivated fungi to competitor fungi,

   and wherein the desired fungi will satisfy both tests;
5) isolation of the desired fungi;

wherein the method may additionally include or comprise the step of identifying the fungi at any time.

Preferably the collection of step 1) occurs on trees and/or wood of the species *Pinus radiata* and/or on trees and/or wood of *Eucalyptus spp*.

Preferably the optional culturing of step 2) occurs axenically through the use of antibiotics such as but not limited to penicillin and/or streptomycin in mixtures of agar and malt extract. More preferably, preparation of the axenic culture of white rot decay fungi is conducted in mixtures of 1-3% agar and 2-5% malt extract.

Preferably the solid cultivation of step 3) occurs between particulate wood including wood chips, sawdust or the like.

Preferably the particulate wood of the solid cultivation of step 3) is *Pinus radiata* or *Eucalyptus spp*.

In a second aspect of the invention there is provided **a biologically pure culture of white rot decay fungi** which will have a positive effect on wood and/or wood products and/or

ood processing by providing one or both of a lignocelluslosic and/or extractives decrease in the wood isolated according to the above method.

In one embodiment, the white rot decay fungi are of the class Basidiomycetes, order Aphyllophorales.

Preferably, said fungi may be selected from the following species:

Ceriporiopsis subvermispora;

Pleurotus ostreatus;

Coriolus versicolor.

Preferably, said fungi may be selected from the following strains:

10-P;

24-P;

15-A.

More preferably the white rot decay fungi is a strain of *Coriolus versicolor* having all the identifying characteristics of the fungi of AGAL Accession Number NM02/32225.

In another embodiment, the white rot decay fungi are of the class Ascomycetes, order Plectoascomycetes.

In a third aspect of the invention there is provided **a biologically pure culture of white rot decay fungi** which when subjected to the steps of:

1) preparation of a solid cultivation of the fungi;
2) subjecting the cultivated fungi to a selection process to distinguish desired fungi from unwanted fungi, wherein the selection process includes or comprises subjecting the cultivated fungi to both:
    a) a test to establish production of oxidative enzymes; and
    b) a test to establish the resistance of the cultivated fungi to competitor fungi,

    and wherein the desired fungi will satisfy both tests;
3) isolation of the desired fungi;

will, when applied to wood, have a positive effect on wood and/or wood products and/or wood processing by providing one or both of a lignocellulosic and/or extractives decrease in the wood.

Preferably the solid cultivation of step 1) occurs between particulate wood including wood chips, sawdust or the like.

Preferably the particulate wood of the solid cultivation of step 1) is *Pinus radiata* or *Eucalyptus spp*.

In a fourth aspect of the invention, there is provided **a method for the preparation of a composition** which will have a positive effect on wood and/or wood products and/or wood processing by providing one or both of a lignocelluslosic and/or extractives decrease in the wood, the method comprising or including the steps of:

a) isolation of white rot decay fungi as disclosed in the above method;
b) use of a reproductively viable form and amount of at least one white rot fungi so isolated, optionally together with one or more agriculturally acceptable carriers, diluents, or adjuvants, in the preparation of a composition.

Preferably step a) of the method includes the preparation between solid wood of the white rot decay fungi, such solid wood being raw wood residuals exemplified by but not limited to chips, sawdust and/or chips. Preferably, the raw wood residuals are but are not limited to chips, sawdust and/or chips of softwoods or hardwoods including *Pinus radiata* or *Eucalyptus spp*.

Preferably the reproductively viable form and amount of the white rot fungi is prepared by massive vegetative reproduction.

Preferably the wood between which the reproductively viable form and amount of the white rot fungi is prepared and the wood used for pulp production to which the composition is to be subsequently applied are from the same species.

In a fifth aspect of the present invention there is provided **a composition which will have a positive effect on wood and/or wood products and/or wood processing** by providing one or both of a lignocelluslosic and/or extractives decrease in the wood, prepared according to the above method.

Preferably the composition has a moisture content of 60 –80%.

It is presently most preferred that the white rot decay fungi is, or the composition includes, *Coriolus versicolor* AGAL Accession Number NM02/32225.

In a sixth aspect of the present invention there is provided **a method of enhancing wood or wood products quality**, the method comprising or including the steps of:

a) preparation of a composition comprising or including white rot decay fungi according to the above method;
b) application of the composition to wood subsequently used for pulp production.

Preferably the wood between which the reproductively viable form and amount of the white rot fungi of the composition is prepared and the wood used for pulp production to which the composition is subsequently applied are from the same species.

Preferably the application of the composition of step b) may be manual and/or automated.

Preferably the composition is applied to logs with or without bark and/or chips.

Preferably the composition is applied to the wood used for pulp production in the forest and/or storing yard and/or mill.

Preferably the composition is applied to the wood used for pulp production at a ratio of between 1 and 4% (w/w) fungi/dry or wet weight of wood.

Preferably the method may further include the step of maintaining the wood source to which the composition has been applied under conditions which allow fungal growth

om the composition for a term sufficient to effect a lignocellulosic and/or extractives decrease. More preferably, the composition is applied to the wood used for pulp production for a period of from 15 days to 4 months.

It is presently most preferred that the white rot decay fungi is, or the composition includes, *Coriolus versicolor* AGAL Accession Number NM02/32225.

In a seventh aspect of the invention there is provided **the wood or wood products** prepared according to the method described above.

In an eighth aspect of the present invention there is provided **a method of improved chemical and/or modified chemical pulping process**, the method comprising or including the steps of:

a) preparation of a composition comprising or including white rot decay fungi by the above described method; and
b) application of the composition to wood subsequently used for pulp production.

Preferably the method provides an increase in pulping efficiency of a chemical and/or modified chemical pulping process, the method being capable of achieving lower kappa numbers with treatment of the wood with white rot decay fungi. Additionally or alternatively the method provides a reduction in pulping energy consumption. Additionally or alternatively the method provides a reduction in pulping chemical processing liquour consumption.

Preferably the wood between which the reproductively viable form and amount of the white rot fungi of the composition is prepared and the wood used for pulp production to which the composition is subsequently applied are from the same species.

Preferably the composition has a moisture content of 60 –80%.

Preferably the application of the composition of step b) may be manual and/or automated.

Preferably the composition is applied to logs with or without bark and/or chips.

Preferably the composition is applied to the wood used for pulp production in the forest and/or storing yard and/or mill.

Preferably the composition is applied to the wood used for pulp production at a ratio of between 1 and 4% (w/w) white rot decay fungi/dry or wet weight of wood.

Preferably the method may further include the step of maintaining the wood source to which the composition has been applied under conditions which allow fungal growth from the composition for a term sufficient to effect a lignocellulosic and/or extractives decrease. More preferably, the composition is applied to the wood used for pulp production for a period of from 15 days to 4 months.

It is presently most preferred that the white rot decay fungi is, or the composition includes, *Coriolus versicolor* AGAL Accession Number NM02/32225.

In a ninth aspect of the invention there is provided **a pulp** prepared according to the method described above.

In a tenth aspect of the invention there is provided **a biologically pure culture of** *Coriolus versicolor* AGAL Accession Number NM02/32225.

In an eleventh aspect, the present invention relates to the use of *Coriolus versicolor* AGAL Accession Number NM02/32225 in a composition, method, or process of the invention.

Description of the Figures

The invention will now be described with reference to the Figures in which:

Figure 1 illustrates a wooden cut to reproduce the white decay fungi (WDF);

Figure 2 illustrates the development of the fungi in a specific selected substrate;

Figure 3 illustrates sample development and antagonism results for different temperatures;

Figure 4 illustrates oxidation intensities of fungi;

Figure 5 illustrates sample of preferable WDF action on the fibres corners and mediates.

Detailed Description of the Invention

The invention includes:

- a process of white rot decay fungi (WDF) development, from an inoculum to solid wood to a composition comprising or including WDF;
- the application of the composition comprising or including WDF produces an initial delignification and extractives decrease of wood, with the purpose of improving the cellulose pulp production via chemical process (either kraft process or sulfite pulp process);
- The wood source is preferably logs, chips, chips and/or sawdust of ***Pinus radiata*** and ***Eucalyptus spp***, but is not limited to these genera or species;

The inventors have undertaken the development of techniques for the selection of fungi, an earlier embodiment of which was called hybridism[10], with a view to optimising the action of certain WDF, with the purpose of selecting desirable characteristics of the fungi. The desirable characteristics are principally:

- the main depolymerization action on lignin, dependent on oxidative enzymes production, such enzymes exemplified by laccase, manganese (Mn) peroxidase and Lignin peroxidase; and
- the resistance of this fungus to antibiosis, that is, resistance to the competition of others that can annul their presence.

The present invention resides in looking for the effect of environmental variables on the behavior of white rot decay fungi to evaluate two variables, these variables being:

1) the production of oxidative enzymes;
2) resistance to competitors.

The following influence the overall efficiency of the white rot decay process of wood and the time of exposure:

1) physical environmental factors, such as moisture content and temperature;
2) chemical factors, for instance the concentration of I oxygenate, carbonic anhydride and the pH;
3) biological factors, for instance the nature of the forest species.

The result of the selection techniques and the positive process effects they convey to wood processing can be summarized as the search of the interaction of the ecological factors to optimize the critical variables, in different white rot decay fungi. The fungi to which the selection techniques can be applied correspond mainly to Basidiomycetes, order Aphyllophorales and in second option Ascomycetes, order Plectoascomycetes.

Inocula Preparation/Fungal Culture

Three means of inocula preparation and culture of fungi exist:

a) liquid - cultivation and propagation of conidia and/or spores and/or somatic body (hyphae) in nutritious liquid solutions;
b) semi-solid - cultivation and propagation utilizing a solid material diluted thoroughly in nutritious liquid solutions; and
c) solid - cultivation and propagation utilizing a solid, relatively humidified material.

Liquid cultivation has been broadly used. Semi-solid cultivation shows little diffusion of the fungi onto the wood and results in a null application. Also, it is necessary to highlight that liquid cultivation and semi-solid cultivation utilize a support enriched with nutrients which allows the colonization of many other fungi different to that of interest, rendering liquid and semi-solid cultivation methods ill-suited to use in uncontrolled atmospheres, such as in a forest or mill.

Solid cultivation has not been previously employed for the purposes described in the present invention.

redelignifcation of Wood

Another purpose of the present invention is the predelignification of solid wood or chips, with the objective of decreasing lignin presence in the pulpwood, and thereby diminishing the chemical digestion required and so minimizing unfavorable environmental impacts.

Thus the present invention also relates to an application process for logs and chips of softwoods and hardwoods (specifically illustrated by examples of ***Pinus radiata*** or ***Eucalyptus spp***) with compositions comprising or including at least one white rot decay fungi, with the main objective being the predigestion of a component of the cellular wall, especially lignin, thereby reducing subsequent chemical cooking (either chemical, time or temperature) in the industrial production of cellulose pulp, improving yields, diminishing requirements of bleaching reagents, making easier the refining of fibers for papers production and improving the properties (mainly tear and tensile energy absorption (TEA)) of same.

The process considers the application of a composition comprising or including white rot fungi with a moisture content about 60 to 80% using woody residuals as chips, chips and/or sawdust of the forest species with the application of composition in manual or automated form, on the pulp wood, which will be constituted by industrial pieces with or without bark and/or chips. This is used for certain forest species, including ***Pinus radiata*** and ***Eucalyptus spp*** but not limited to these species or genera.

Also, the application can be directly in the forest, or mills. The intensity of application of the composition can vary, between 1 and 4%, weight of white rot decay fungi to weight of the woody material.

The time of predelignification of forest species treated with the composition varies from 15 days to 4 months, dependent upon the bioclimatic situation, the treatment conditions, the forest species and/or the quality of the product, and considering if they are logs with or without bark, chips, and so on.

he predelignification of wood of the present invention is achieved by means of:

- the isolation of the fungi of white rot decay, to prepare an inoculate between solid residues of white rot decay fungi on compost comprising such wood residuals as chips, chips and/or sawdust of softwood and/or hardwood including but not limited to ***Pinus radiata*** or ***Eucalyptus spp***; and
- the use of the inoculate in the preparation of a composition, wherein the inoculate is prepared on solid wood residuals;
- the application of the composition on the forest species to be used later for cellulose pulp production,

whereby the application of the composition comprising or including white rot decay fungi results in the digestion of part or all of the lignocellulose fraction in the wood.

Process

A non-limitative application example of the invention is now described. The procedure for obtaining a solid cultivation requires the following steps:

a) Collection and identification of fungi in nature, whereby fungal material is isolated in form of sexual bodies from infected lignocellulosics, and later in the laboratory identified by the use of appropriate taxonomic key;

b) Isolation and cultivation in mixtures of Agar and of Extract of Malt, more specifically between 1-3% Agar and 2-15% Extract of Malt, in any form, by means of the use of mixtures of antibiotics such as penicillin and streptomycin;

c) Application of the Noblemen method to determine the presence of oxidative enzymes;

d) Determination of the resistance to antibiosis exhibited by the fungi;

e) Preparation of the nutritious support for fungal multiplication;

f) Large-scale vegetative reproduction of the selected fungi.

Example

The following non-limitative example of the invention is presented to illustrate the steps of the inventive process of the invention, the results of the application of white rot decay fungi in the processes of predelignification of pulpwood, and the results of the final product of the pulping process.

a) Isolation: wooden pieces with white rot decay fungi are sowed principally in cultivation of Agar-Malt with addition of bacterial antibiotics as is illustrated in Figure 1. Once mycelia formation is achieved, the fungi is purified by standard microbiology techniques.

b) Preparation of the reproductively viable form and amount of white rot fungi between solid wooden residuals: wooden residuals as chips, chips and/or sawdust of the forest species to be subsequently treated in the predelignification process with a variable moisture content between a 60 and 80%, in either a natural state or previously sterilized, are inoculated with the selected white rot decay fungi, as is shown in Figure 2.

c) Tests of antibiosis, in the presence of competitive organisms: the white rot decay fungus elect is cultivated under conditions of defined pH, and contacted with an infection fungi which is commonly present in the natural environment. This occurs at different temperatures, as is illustrated in Figure 3. The results and later analysis allow determination of the environmental factors under which the test fungi provides the highest resistance to antagonisms and/or undesired parasitisms.

d) Tests of oxidative enzymes: Principal cultivation, based on tannins and Agar and the production of oxidative enzymes of the fungi of white rot decay is determined, wherein the level of oxidation is revealed by the intensity of the brown coloration and the radius of the aureole surrounding the fungi, as illustrated in Figure 4. Cultivation is carried out at different incubation temperatures.

e) Composition application on Wood: Once grown on the substrate (sawdust, chip and/or splinters, etc), the white rot decay fungi goes through a period of maturation in the

boratory of approximately 1-5 weeks, after which the composition can be applied in quantities of 1-4% (w/w fungi/wood) over industrial logs or chips of softwoods or hardwoods such as ***Pinus radiata*** or ***Eucalyptus spp.***

f) Lignin removal in the woody cell: In Figure 5 the oxidation of the lignin is shown, particularly in the middle lamella, as observed in the cellular vertexes. In both portions of the cellular wall, this is where the biggest quantity of highly condensed lignin exists.

In Table I below the effect of environmental factors is shown in relation to levels of oxidative enzymes production in Units of enzyme accumulated in extracellular growth medium/ml:

Table I

WDF fungal isolates	Temperature	Laccase U/ml	Mn Peroxidase U/ml
9-C	20°C	0.080	0.160
	25°C	0.130	0.120
	30°C	0.140	0.110
10-P	20°C	1.500	0.240
	25°C	1.000	0.120
	30°C	0.260	0.020
24-P	20°C	0.410	0.026
	25°C	1.810	0.063
	30°C	1.670	0.073
9-P	20°C	1.770	0.320
	25°C	0.220	0.031
	30°C	1.890	0.250
15-A	20°C	0.120	0.043
	25°C	0.060	0.028
	30°C	0.270	0.051

The white rot decay fungi presented in Table I correspond to species of Basidiomycetes, order Aphyllophorales and the applicable isolate designation. The fungi were isolated at different environmental development conditions such as temperature, pH, concentration

of carbonic anhydride, etc. The results, expressed as concentration of oxidative enzymes, demonstrate the influence of temperature as a selection variable.

In Table II the bleached pulps results are shown for wood being ***Pinus radiata***, pretreated with white rot decay fungi.

Table II

	Yield %	**Kappa**	**Kappa after O_2**	**Delta Kappa**	**Yield after O2 %**
Control	47.1	29.4	14.0	52.4	96.1
9 P	49.5	31.5	21.1	33.0	97.5
9 C	49.2	31.8	20.8	34.5	97.8
24 P	49.8	30.7	20.9	31.9	99.0

	Act. Chlor. CD	**Viscos. mPa s**	**Yield %**	**Brightness**	**Total Cl**
Control	0.3	9.7	91.3	89.6	2.8
9 P	0.2	16.7	92.5	92.6	2.8
9 C	0.2	15.7	92.1	92.0	2.79
24 P	0.2	18.5	94.0	92.6	2.78

The term kappa is the characterization of the residual lignin in raw kraft pulp, the yield is expressed in dry weight of pulp on the base of wood dry weight that enters the pulping process. The kappa after oxygen bleaching stage (O_2) corresponds to the value of the index after having submitted the pulp to delignification with O_2. Delta Kappa corresponds to how much lower the kappa with delignification by O_2 results, and the yield is as above but expressed in dry weight of O_2 bleached pulp over dry weight of the raw pulp as it entered the O_2 stage in %.

The control corresponds to the natural wood of the same species, in this case ***Pinus radiata***, which was not pretreated with the composition including or comprising White Rot Decay Fungus.

As is observed in Table II, and as previously discussed, the pulp yield of the wood treated with WDF, is 5-6 % more than in the control (non-fungal treated wood pulp). With smaller rates of chlorine or equivalent of chlorine, or any other oxidative bleach chemical, the samples of cellulose pulps derived of biotreated wood have better yield after the process of bleaching and higher final brightness, 3-5 % higher than the control.

The physical-mechanical properties of bleached papers are shown in Table III.

Table III

PULP	PFI REV	Free. CSF	Tear I.	Tensile	T.E.A.	Brightness
BP-CH 10D	1000	684	14.20	071.6	1309	87.7
	2000	657	11.28	085.8	1638	84.0
	4000	549	09.74	101.7	2100	83.3
	8000	260	08.46	110.6	2260	78.6
BP-CH11B	1000	651	10.76	075.8	1779	85.4
	2000	631	09.30	090.0	2095	84.6
	4000	542	08.10	102.5	2308	84.4
	8000	309	07.71	111.0	2506	82.4
BP-CH12A	1000	694	12.30	078.2	1596	87.1
	2000	663	09.72	093.2	1973	86.1
	4000	554	08.92	102.7	2218	84.4
	8000	308	07.98	112.1	2357	83.1
CONTROL	1000	630	08.58	059.5	1220	84.9
	2000	567	07.33	070.2	1458	83.9
	4000	415	06.15	078.7	1562	82.0
	8000	135	05.34	087.0	1740	76.6

The term freeness corresponds to the measure of the capacity of pulp for retaining water. Tear Index indicates the resistance of paper to suffer a rupture in the border of paper. Tensile indicates the resistance of pulp to the traction or tension, TEA corresponds to tensile energy absorption, in another way, the resistance expressed by the internal

ohesion of paper (fibres unions). Brightness is the capacity of paper to reemit the light that impacts on paper.

For all the refining levels (PFI rev.), the properties of papers derived from biopulps (BP-CH IOD, BP-CHIIB, BP-CH12A) are of better characteristics that the control samples. The freeness of pulps is improved. The tear indices for all the biotreatments are better, by at least 38%. The tensile indices of biopulps are greater by 24-36%. Biopulp TEA indices are increased by 12-18%. The brightness is 2-4 % higher in biopulps in relation to the control.

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DATED THIS 24th DAY OF April 2003
AJ PARK
PER
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24 APR 2003

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